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Inhibition of Pro-inflammatory Secreted Phospholipase A2 by Extracts from *Cynara cardunculus L.*

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Abstract The aim of the present work was to evaluate the anti-inflammatory properties of *Cynara cardunculus* L. (Asteraceae) during its growth using various solvents such as *n*-hexane, dichloromethane, acetone, and methanol for air-dried leaves and stems. The anti-inflammatory activities of crude extracts were evaluated by measuring the inhibition potency of mammalian non-pancreatic phospholipases A2 (hG-IIA). The methanol and acetone extracts of leaves harvested in February exhibit potent inhibition of hG-IIA (IC_{50}= 50 and 70 μg/ml, respectively). However, the acetone extract of stems harvested in December inhibits the hG-IIA with a lower IC_{50} around 130 μg/ml. Fractionation on silica gel and hydrophobic gel of the methanol extract of leaves harvested in February increases the inhibitory effect, and the IC_{50} reached 10 μg/ml.

Keywords Enzyme · Secreted phospholipases A2 · Extraction · Inhibitors · Anti-inflammatory · *Cynara cardunculus*

Introduction

Phospholipases A2 (PLA2, EC 3.1.1.4) are a family of enzymes that catalyze the hydrolysis of glycerophospholipids at the *Sn-2* position, producing free fatty acids and lysophospholipids [1–3]. The mammalian family of PLA2 includes the secreted forms (sPLA2) which are 14–19 kDa, Ca^{2+} dependent, disulfide-rich enzymes. PLA2s participate in cellular eicosanoid metabolism and signal transduction. Ten human secreted PLA2s have been cloned: group (G) IB, GIIA, GIID, GIIE, GIIF, GIII, GV, GX, and GXIIA PLA2s and the GXIIB PLA2-like protein that is devoid of catalytic activity [4, 5]. GIB PLA2 is referred to as "pancreatic" PLA2 since it is abundantly expressed in pancreatic acinar cells [6, 7]. The

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GIB PLA2 gene was cloned in 1986 [8]. In 1989, GIIA PLA2 was sequenced and cloned from material obtained from rheumatoid arthritic synovial fluid [9] and blood platelets [10]. The enzyme is called "nonpancreatic" or "synovial" PLA2. The enzymes belonging to group IIA play important role in the initiation and amplification of the inflammatory reaction by the liberation of arachidonic acid from the membrane phospholipids and in the biosynthesis of eicosanoids from various lipid mediators produced elsewhere [11–15]. Group IIA sPLA2s are also known as the inflammatory-type sPLA2 since they are highly expressed in the plasma and synovial fluids of patients with various inflammatory diseases such as rheumatoid arthritis, acute pancreatitis, Crohn's disease and endotoxic shock [3, 16–18] as well as in various cancers [19, 20]. The pro-inflammatory actions of sPLA2-IIA in local inflammatory processes have been verified by injecting either sPLA2-IIA [21, 22] or its inhibitors or antibodies [23, 24] to animals suffering from various types of inflammation. Actual anti-inflammatory therapies include non-steroidal anti-inflammatory drugs that inhibit the cyclooxygenase-1 or -2 (COX-1/2) enzymes and then prevent the conversion of arachidonic acid to prostaglandins. However, most of all these drugs have some limitations and cannot regulate the production of leukotrienes or platelet-activating factor (PAF) that continues to cause inflammation [25, 26]. It appears rational that effective inhibitors of PLA2 could deplete the sources of arachidonic acid and, therefore, its downstream metabolites and PAF, without affecting the COX1/COX2 homeostasis. The potential value of PLA2 inhibitors as anti-inflammatory drugs was recognized during the 1980s by many major pharmaceutical companies that undertook inhibitor development programs [27]. We can mention the YM26374 by Yamanouchi Pharma [28] and pentapeptide inhibitors claimed by the Garvan Institute of Medical Research. Moreover, Eli Lilly's and Shionogi have developed several (indole-3-yl) oxoacetamile structurally related to LY315920 [27] and Indoxam [29].

The plant *Cynara cardunculus*, commonly named cardoon, is known for its therapeutical uses in folklore as diuretic and choleretic activities [30, 31]. Previous chemical investigations demonstrated the presence of saponins [32], sesquiterpene [33], lactones [34], and lignans [35] in this plant. Moreover, many aspartic proteases are also present in *C. cardunculus*. They are known as cardiosins, cyprosins, cenprosins, and cynarases [36–40]. The aim of this study was to evaluate the anti-inflammatory properties of different extracts of *C. cardunculus L.* during its growth by measuring the inhibitory effects on the hG-IIA phospholipase A2 using various solvents for the extraction.

Materials and Methods

Plant Samples

The leaves and the stems of *C. cardunculus* L. were collected in the region of Sfax (South of Tunisia) in December, February, and May. The plant was identified by Prof. M. Chaieb (FSS, Tunisia), and a voucher specimen has been deposed in the Natural Substance Chemistry Laboratory, Faculty of Sciences of Sfax, Tunisia.

Extractions

Dried and powdered leaves and stems were extracted successively using a soxhlet apparatus with *n*-hexane, dichloromethane, acetone, and methanol. All extracts were concentrated to dryness in vacuo and stored in the dark at 4 °C until use.

Enzyme and Reagents

hG-IIA and Me-Indoxam were generous gifts from Dr Gérard Lambeau (IPMC, France).

pG-IB, egg yolk phosphatidylcholine, red phenol, and sodium taurodeoxycholate (NaTDC) were purchased from Sigma Chemical (USA).

Phospholipase Inactivation

Two methods were used to determine the inhibitory effect of extracts and fractions depending upon the order of addition of phospholipase, substrate, and inactivator.

Method A: phospholipase/inactivator pre-incubation method. This method was set up to test the possible reaction between phospholipase and inactivator in the absence of substrate.

Method B: inactivation during lipolysis. This method was designed to test whether any inactivation reaction occurred in the presence of substrate during phospholipids hydrolysis.

These two methods can give us an idea about the nature (hydrophobic or lipophilic) of the inhibitor compound [41]. In fact, if the phospholipase activity decrease when using method A (pre-incubation inhibitor enzyme), the inhibitor may acts as a pseudo-substrate and is able to form an interface [42]. The enzyme binds to this "poisonous" interface and will be inhibited. If there is no inhibition when method B is used, it could mean that the inhibitor is water-soluble and cannot reach the enzyme which is bound to the substrate–water interface [42]. The enzyme is not affected and continues to hydrolyze its substrate.

Colorimetric Assay of sPLA2 Activity

sPLA2 (IIA and IB) activities were measured by colorimetry at 558 nm [43]. Egg yolk phospholipids (3.5 mM) were suspended in 0.1 M NaCl, 0.01 M $CaCl_2$, 3 mM NaTDC, and 0.055 mM red phenol, pH 7.6. PLA2 activity was expressed in international units; one phospholipase unit corresponds to 1 μmol of titratable released fatty acid per minute under described conditions. For inhibition experiments, sPLA2 (0.2 μg) was mixed and incubated at room temperature for 20 min with variable amounts of crude extracts before the measurement of PLA2 residual activity. Control experiments were performed with the corresponding amounts of solvent without extract. The residual activity of sPLA2 and the IC_{50} values were then determined. The positive control measurement was carried out with Me-indoxam known as a potent covalent inhibitor of sPLA2.

Fractionation of Compounds

One gram of methanol extract from leaves, February harvest, was applied on a silica gel column. A mixture of chloroform/methanol in different volume ratios allowed the elution (0:1, 1:4, 2:3, 3:2, 4:1, and 1:0). Fractions results were labeled 1–6, evaporated, and then tested for PLA2 inhibitory effect. Fraction 2 (109 mg) was found to be the most active fraction. This fraction was applied on a medium pressure reverse phase column (C8) using acetonitrile/water (1:4, 1:1, and 1:0). To obtain the chromatography, the column was washed with methanol. Four fractions were collected, evaporated, labeled 2a–2d, and again tested for their inhibitory effect on PLA2.

Statistical Analysis

All the results related to the determination of the extract inhibitory effects were the average of three replicates of analysis. They were statistically analyzed by SPSS software using Duncan test performed after analysis of variance.

Results

Extraction Yields of Plant Material

Leaves and stems of *C. cardunculus* were collected in different months (December, February, and May). They were harvested and extracted by different solvents (*n*-hexane, dichloromethane, acetone, and methanol). The extraction yields are reported in Table 1.

Evaluation of the Inhibitory Effect of Leaves

The inhibitory effect of leaves was evaluated using method A as described in "Materials and Methods." Results reported in Fig. 1 show that *n*-hexane and dichloromethane extracts of leaves did not show any significant inhibitory effects on the hG-IIA phospholipase, but a

Table 1 Extraction yields of plant material with various solvent.

Organs	Month of harvest	Solvent of extraction	Yield (g/kg)
Leaves	December	Hexane	23.0
		CH_2Cl_2	36.0
		Acetone	24.2
		MeOH	57.9
	February	Hexane	14.8
		CH_2Cl_2	26.8
		Acetone	15.5
		MeOH	36.0
	May	Hexane	27.7
		CH_2Cl_2	44.4
		Acetone	31.5
		MeOH	57.7
Stems	December	Hexane	9.7
		CH_2Cl_2	9.8
		Acetone	10.3
		MeOH	44.2
	February	Hexane	7.6
		CH_2Cl_2	7.5
		Acetone	4.4
		MeOH	59.4
	May	Hexane	22.4
		CH_2Cl_2	9.1
		Acetone	5.0
		MeOH	142.0

The same volume for each solvent was used for extraction which is about 1.5 l for 250 g of dried plant

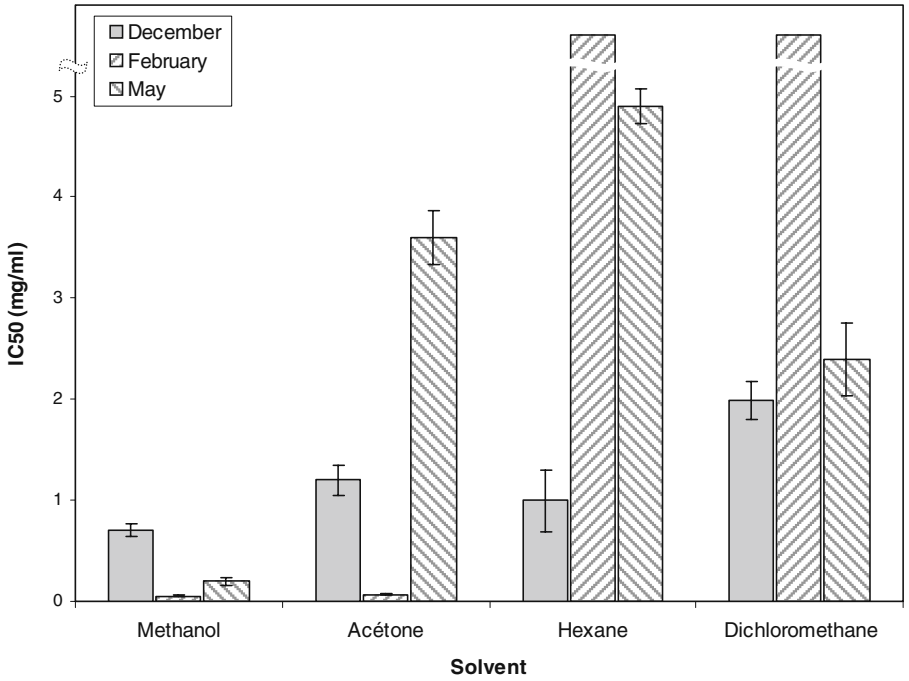

Fig. 1 Evaluation of anti-PLA2 activity of leaves. Three harvests of air leaves of *C. cardunculus* L. were carried out in different months: the methanol extract of leaves harvested in February has an IC_{50} value of 50 μg/ml, and the one of acetone extract is of 70 μg/ml. All experiments were performed in triplicate

significant inhibitory difference effect can be observed on harvest of a different month. However, acetone and methanol extracts showed good inhibitory effects. The most significant inhibitions, corresponding to an IC_{50} value of 50 and 70 μg/ml for methanol and acetone extracts, respectively, were observed in February harvest.

Evaluation of the Inhibitory Effect of Stems

The inhibitory effect of stems was evaluated using the same method A as previously described. Figure 2 shows that the extracts obtained using *n*-hexane and dichloromethane have no inhibitory effect on hG-IIA phospholipase. However, inhibition was observed in acetone and methanol extracts. In fact, the methanol extract inhibits hG-IIA with an IC_{50} of 300 μg/ml when stems are harvested in February and 200 μg/ml when they are harvested on May. Meanwhile, acetone extracts shows an IC_{50} value of 130 and 180 μg/ml when stems are collected on December and February, respectively.

Methanol extract of leaves collected in February with IC_{50} of 50 μg/ml on hG-IIA was chosen as the best fraction, and we evaluated their effect on the pancreatic sPLA2 (pG-IB). Our results demonstrate that these extracts inhibit pG-IB with an IC_{50} value of 500 μg/ml. We can conclude that inhibitory effect is more selective for hG-IIA more than for pG-IB. We used the methanol extract of dry leaves harvested in February for fractionation. During purification, fractions were tested for the inhibitory effect on hG-IIA and pG-IB. Fraction 2 eluted from silica gel, which has an IC_{50} of 50 μg/ml, was subjected to a C8 chromatography. The elution from this hydrophobic column was performed using a

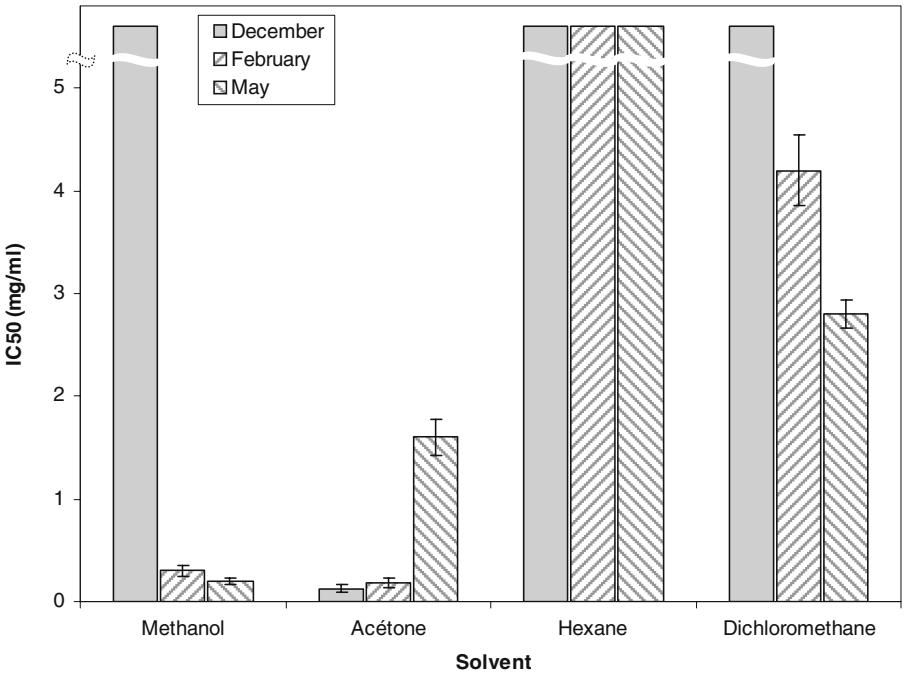

Fig. 2 Evaluation of anti-PLA2 activity of stems. Three harvests of stems of *C. cardunculus* L. were carried out in different months. The acetone extract of stems harvested in December and February show a potent inhibition with an IC_{50} of 130 and 180 μg/ml, respectively. The reference molecule used on inhibitory test was the Me-Indoxam. In the same conditions, the IC_{50} of inhibition effect of the Me-Indoxam on hG-IIA is 0.185 μg/ml (0.4 μM). All experiments were performed in triplicate

mixture of acetonitrile/water. The most potent inhibitory effects were observed with fractions eluted at an acetonitrile/water, 1:1 and 1:0, respectively. The IC_{50} of these fractions are 10 and 20 μg/ml, respectively (data not shown).

Evaluation of the Inhibitory Effect Using Method B

This method is used to estimate the potency of inhibition during the substrate hydrolysis. The enzyme (hG-IIA) was added to the reaction mixture, then after few minutes, we put in the inhibitor when the enzyme underway substrate. Residual activity was then evaluated.

As shown in Fig. 3, the IC_{50} of fraction 2b was 17 μg/ml. This result demonstrates that to inhibit phospholipases A2 using method B, we are obliged to use a higher amount of inhibitor then in method A. Moreover, we can conclude that the molecule responsible for the inhibitory effect is hydrosoluble and cannot reach the lipidic interface when the enzyme is adsorbed. In fact, tetrahydrolipstatin, a lipase inhibitor, is liposoluble, and the concentration used to inhibit pancreatic lipase using method A is roughly the same as in method B [44].

Discussion

To evaluate the anti-inflammatory effect of C. *cardunculus* on the biosynthesis of inflammatory mediators, different extracts were used to inhibit human pro-inflammatory

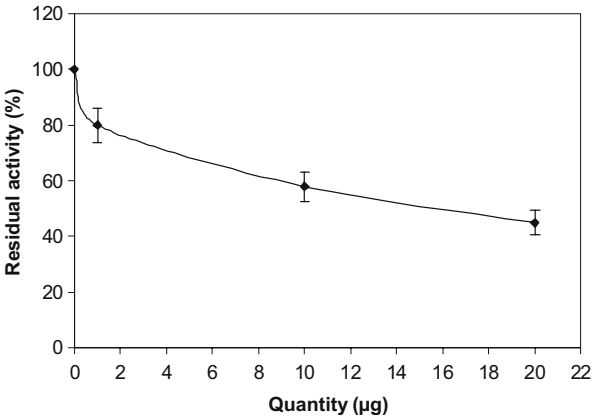

Fig. 3 Evaluation of PLA2 inhibition during the substrate hydrolysis. Determination of the capacity of fraction 2b to inhibit the PLA2 during hydrolysis shows that the IC_{50} was around 17 µg/ml. All experiments were performed in triplicate

PLA2. Results of this study showed that the percentage of inhibition depends on the nature of solvent used for extraction. In fact, we showed that the inhibitory effect of extracts increases with the polarity of the solvent used. This is may be due to the presence of polar compounds such as lignans which are known for their anti-inflammatory activities [45]. When polar solvents were used for the extraction such as acetone and methanol, differences on inhibitory effect were observed between leaves and stems. However, leaf extracts have more capacity to inhibit hG-IIA phospholipase A2 than stems when using methanol as extraction solvent whatever the month of harvesting. Using acetone for extraction, only the leaf extracts harvested in February show a higher inhibitory effect than that of stem extracts (IC_{50} of 70 and 180 µg/ml, respectively). However, extracts of stems obtained in December (IC_{50} of 130 µg/ml) and May (IC_{50} of 1.6 mg/ml) show a better potency to inhibit sPLA2 than those of leaves (IC_{50} of 1.2 and 3.6 mg/ml, respectively). In conclusion, methanol and acetone used for extraction of leaves harvested in February and the acetone extract of steams harvested in December showed a potent inhibition of human sPLA2 involved in the inflammatory processes.

Several studies investigated medicinal plants for potent natural inhibitors. From nine vine plants used in traditional Chinese medicine to treat inflammations, three of them exhibit a capacity to inhibit the PLA2 activity [46]. The IC_{50} values obtained with ethanol extract of the stem of *Sinomenium acutum*, *Spatholobus suberectus*, and *Trachelospermum jasminoide* were 112, 54, and 33 µg/ml, respectively [46]. Other study shows that the aqueous extract of *Rhizophora* mangle bark inhibited sPLA2 with an IC_{50} value of 72 µg/ml [47]. Nunez et al. [48] have isolated 4-nerolidylcatechol as an active principle that inhibit secreted phospholipase A2 group II. The enzyme activity of *Bothrops asper* myotoxin I was completely inhibited by 4-nerolylcatechol at an inhibitor/toxin ratio of 10:1 (wt/wt) with an IC_{50} of around 1 mM. Compared to these works, our study on *C. cardunculus* extracts seems interesting.

Purification and identification of sPLA2 inhibitors from *C. cardunculus* extracts are under investigation. Pharmacological study of the extract in animals is of high interest to develop new anti-inflammatory drugs.

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References

1. Dennis, E. A. (1994). *Journal of Biological Chemistry, 269*, 13057–13060.
2. Dennis, E. A. (1997). *Trends in Biochemical Sciences, 22*, 1–2.
3. Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., & Kudo, I. (1997). *Critical Reviews in Immunology, 17*, 225–283.
4. Six, D. A., & Dennis, E. A. (2000). *Biochimica et Biophysica Acta, 1488*, 1–19.
5. Rouault, M., Bollinger, J. G., Lazdunski, M., Gelb, M. H., & Lambeau, G. (2003). *Biochemistry, 42*, 11494–11503.
6. Eskola, J. U., Nevalainen, T. J., & Aho, H. J. (1983). *Clinical Chemistry, 29*, 1772–1776.
7. Nevalainen, T. J., & Haapanen, T. J. (1993). *Inflammation, 17*, 453–464.
8. Seilhamer, J. J., Randall, T. L., Yamanaka, M., & Johnson, L. K. (1986). *DNA, 5*, 519–527.
9. Seilhamer, J. J., Pruzanski, W., Vadas, P., Plant, S., Miller, J. A., Kloss, J., et al. (1989). *Journal of Biological Chemistry, 5*, 5335–5338.
10. Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., et al. (1989). *Journal of Biological Chemistry, 264*, 5768–5775.
11. Fourcade, O., Simon, M. F., Viode, C., Rugani, N., Leballe, F., Ragab, A., et al. (1995). *Cell, 80*, 919–927.
12. Balsinde, J., & Dennis, E. A. (1996). *Journal of Biological Chemistry, 271*, 6758–6765.
13. Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M. V., Tischfield, J. A., et al. (1998). *Journal of Biological Chemistry, 273*, 14411–14423.
14. Murakami, M., Koduri, R. S., Enomoto, A., Shimbara, S., Seki, M., Yoshihara, K., et al. (2001). *Journal of Biological Chemistry, 276*, 10083–10096.
15. Bezzine, S., Koduri, R. S., Valentin, E., Murakami, M., Kudo, I., Ghomashchi, F., et al. (2000). *Journal of Biological Chemistry, 275*, 3179–3191.
16. Nevalainen, T. J. (1993). *Clinical Chemistry, 39*, 2453–2459.
17. Vadas, P., Browning, J., Edelson, J., & Pruzanski, W. (1993). *Journal of Lipid Mediators, 8*, 1–30.
18. Mukherjee, A. B., Miele, L., & Pattabiraman, N. (1994). *Biochemical Pharmacology, 48*, 1–10.
19. Murata, K., Egami, H., Kiyohara, H., Oshima, S., Kurizaki, T., & Ogawa, M. (1993). *British Journal of Cancer, 68*, 103–111.
20. Abe, T., Sakamoto, K., Kamohara, H., Hirano, Y., Kuwahara, N., & Ogawa, M. (1997). *International Journal of Cancer, 74*, 245–250.
21. Murakami, M., Kudo, I., Nakamura, H., Yokoyama, Y., Mori, H., & Inoue, K. (1990). *FEBS Letters, 268*, 113–116.
22. Bomalaski, J. S., Lawton, P., & Browning, J. L. (1991). *Journal of Immunology, 146*, 3904–3910.
23. Kakutani, M., Murakami, M., Okamoto, H., Kudo, I., & Inoue, K. (1994). *FEBS Letters, 339*, 76–78.
24. Fleisch, J. H., Armstrong, C. T., Roman, C. R., Mihelich, E. D., Spaethe, S. M., Jackson, W. T., et al. (1996). *Journal of Pharmacology and Experimental Therapeutics, 278*, 252–257.
25. FitzGerald, G. A. (2003). *Naturalist Review Drug Discov, 2*, 879–890.
26. Vane, J. R. (2002). *Science, 296*, 474–475.
27. Reid, R. C. (2005). *Current Medicinal Chemistry, 12*, 3011–3026.
28. Hamaguchi, K., Kuwata, H., Yoshihara, K., Masuda, S., Shimbara, S., Oh-ishi, S., et al. (2003). *Biochimica et Biophysica Acta, 1635*, 37–47.
29. Boilard, E., Rouault, M., Surrel, F., Le Calvez, C., Bezzine, S., Singer, A., et al. (2006). *Biochemistry, 45*, 13203–13218.
30. Lemordant, D., Boukef, K., & Ben Salem, M. (1977). *Fitoterapia, 5*, 191–214.
31. le Floc'h, E. (1983). In *Imprimerie Officielle de la République Tunisienne*, p. 263.
32. Shimizu, S., Ishihara, N., Umehara, K., Miyase, T., & Ueno, A. (1988). *Chemical and Pharmaceutical Bulletin (Tokyo), 36*, 2466–2474.
33. Grancai, D., Mucaji, P., Nagy, M., & Ubik, K. (1996). *Farmaceuticky Obzor, 65*, 255–256.
34. Grancai, D., Nagy, A., Mucaji, P., Suchy, V., & Ubik, K. (1994). *Farmaceuticky Obzor, 63*, 447–449.
35. Koubaa, I., Damak, M., McKillop, A., & Simmonds, M. (1999). *Fitoterapia, 70*, 212.
36. Ramalho-Santos, M., Pissarra, J., Verissimo, P., Pereira, S., Salema, R., Pires, E., et al. (1997). *Planta, 203*, 204–212.
37. White, P. C., Cordeiro, M. C., Arnold, D., Brodelius, P. E., & Kay, J. (1999). *Journal of Biological Chemistry, 274*, 16685–16693.
38. Domingos, A., Cardoso, P. C., Xue, Z. T., Clemente, A., Brodelius, P. E., & Pais, M. S. (2000). *European Journal of Biochemistry, 267*, 6824–6831.
39. Roserio, L. B., Barbosa, M., Ames, J. M., & Wilbey, R. A. (2003). *International Journal of Dairy Technology, 56*, 76–85.

40. Sidrach, L., García-Cánovas, F., Tudela, J., & Rodríguez-López, J. N. (2004). *Phytochemistry, 66*, 41–49.
41. Gargouri, Y., Chahinian, H., Moreau, H., Ransac, S., & Verger, R. (1991). *Biochimica et Biophysica Acta, 1085*, 322–328.
42. Gargouri, Y., Ransac, S., & Verger, R. (1997). *Biochimica et Biophysica Acta, 1344*, 6–37.
43. Lobo de Araujo, A., & Radvanyi, F. (1987). *Toxicon, 25*, 1181–1188.
44. Ransac, S., Gargouri, Y., Marguet, F., Buono, G., Beglinger, C., Hildebrand, P., et al. (1997). *Methods in Enzymology, 286*, 190–231.
45. Kupeli, E., Erdemoglu, N., Yesilada, E., & Sener, B. (2003). *Journal of Ethnopharmacology, 89*, 265–270.
46. Li, R. W., David, G., Myers, S. P., & Leach, D. N. (2003). *Journal of Ethnopharmacology, 85*, 61–67.
47. Marrero, E., Sanchez, J., de Armas, E., Escobar, A., Melchor, G., Abad, M. J., et al. (2006). *Fitoterapia, 77*, 313–315.
48. Nunez, V., Castro, V., Murillo, R., Ponce-Soto, L. A., Merfort, I., & Lomonte, B. (2005). *Phytochemistry, 66*, 1017–1025.